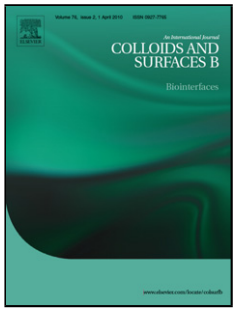

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PII: S0927-7765(17)30309-0
DOI: http://dx.doi.org/doi:10.1016/j.colsurfb.2017.05.051
Reference: COLSUB 8580

To appear in: *Colloids and Surfaces B: Biointerfaces*

Received date: 20-2-2017
Revised date: 28-4-2017
Accepted date: 20-5-2017

Please cite this article as: Rafael A.Bini, Mônica F.Silva, Laudemir C.Varanda, Marcelo da Silva, Cécile A.Dreiss, Soft nanocomposites of gelatin and poly(3-hydroxybutyrate) nanoparticles for dual drug release, Colloids and Surfaces B: Biointerfaceshttp://dx.doi.org/10.1016/j.colsurfb.2017.05.051

Soft nanocomposites of gelatin and poly(3-hydroxybutyrate) nanoparticles for dual drug release

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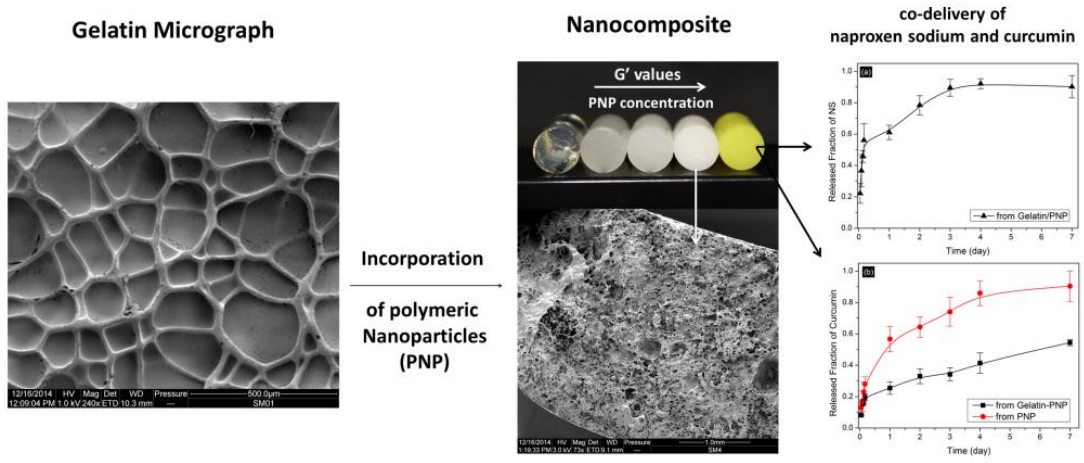
Graphical Abstract



Highlights

- Injectable nanocomposite gels were developed for the co-delivery of two drugs.
- Drugs with opposite solubility were successfully loaded in the nanoparticles and matrix.
- The DDS is based on renewable polymers and gelation obtained by enzymatic cross-linking
- 40% curcumin was released over 4 days, compared to 82% from the free nanoparticles

Abstract

We developed a nanocomposite gel composed of gelatin and poly(3-hydroxybutyrate) polymeric nanoparticles (PNP) to be used as an injectable gel for the contemporaneous, dual sustained release of bioactive molecules. The hydrogel matrix was formed from a very simple process, using either the physical gelation of gelatin or the natural enzyme transglutaminase to covalently cross-link the gelatin chains in the presence of embedded PNP. Oscillatory rheological measurements showed that the addition of the PNP induced an increase in the storage modulus compared to pure gelatin gels, for both physical and chemical gels. Micrographs from scanning electron microscopy revealed that the presence of PNP disrupted the native structure of the gelatin chains in the hydrogel matrix. Dual drug encapsulation was achieved with curcumin (CM) in the PNP and naproxen sodium in the gelatin matrix. *In vitro* release studies showed that the hydrogel matrix acts both as a physical and chemical barrier, delaying the diffusion of the drugs. An initial burst release was observed in the first hours of the measurement, and around 90% was released on the third day for naproxen sodium. In free PNP, 82% of curcumin was relased after four days, while when PNP were embedded in the gelatin matrix only 40% was released over the same time period. Overall, these simple, sustainable soft nanocomposites show potential as an injectable co-sustained drug release system.

Keywords: gelatin hydrogels; poly(3-hydroxybutyrate) nanoparticles; soft nanocomposites; curcumin; naproxen sodium; dual drug delivery.

1. Introduction

The development of effective drug delivery systems (DDS), which help optimise the drug therapeutic effect, while minimizing undesirable side effects, has been the main focus of pharmaceutical research over the past couple of decades. A major challenge faced by the pharmaceutical industry is the increasing lack of solubility of most drug candidates being discovered and the increasing demand to solubilise the drug in an aqueous environment, not only for administration, but also for drug development [1]. In addition to improved solubilisation, the DDS should improve the pharmacokinetics of the drug, prolong circulation times, protect the drug from degradation (chemical, enzymatic, etc), enhance its bioavailability and provide sustained release kinetics [2]. In parallel, the growing interest in biopharmaceuticals (such as recombinant proteins and antibodies) demands the use of 'soft' drug delivery matrices that are able to preserve the inherent characteristics of the biological molecules (preserving the ternary structure, preventing aggregation etc). The advent of nanotechnology has led to the development of a range of suitable DDS, such as polymeric nanoparticles, micelles, liposomes or hydrogels [3-7], which, in addition to the aforementioned properties, often present responsiveness to external triggers, such as temperature or pH, thus affording controlled release and/or loading from the carrier. Recently, the increased understanding of cellular mechanisms, identification of multitarget drugs, and the discovery of synergistic effect achieved by combining multiple drugs, has led to the development of combination therapy, namely, DDS that combine different drugs, thus offering improved efficiency[2, 8]. This area is particularly relevant to biopharmaceuticals, which often need to be combined to a small-drug, and to the cancer arena [2, 8], where combination chemotherapy is becoming increasingly important to maximise therapeutic effect while overcoming drug resistance. The combination of anti-inflammatory and anti-cancer drugs is very promising for cancer therapy because inflammation can affect many aspects of tumour development and progression, as well as the response towards the therapy [9, 10]. One of the main challenges of combined therapy is to control both the loading and the release profile of each drug independently [9-14], and the task is further complicated if the therapeutic molecules have vastly different solubility, for instance one is predominantly hydrophilic and the other hydrophobic. This requires the use of compartmentalised DDS, with solubilisation loci suitable for drugs with vastly different characteristics. On this basis, we report here the design of a DDS for combination therapy, based on polymeric nanoparticles embedded in a hydrogel matrix. The focus is on the use of sustainable materials and simple, low-cost preparation protocols.

Hydrogels, usually based on a network of cross-linked polymeric chains, offer a hydrophilic three-dimensional structure, mechanical properties and an aqueous environment compatible with living tissues, and enable the encapsulation and controlled release of hydrophilic therapeutic agents[15-17]. The combination of such a matrix with polymeric nanoparticles – in so-called 'soft nanocomposites' or 'nanocomposite gels' (NC)[18] – offers a simple, compartmentalised DDS, with potential for combination therapy. The association of a soft hydrophilic matrix with nanoparticles indeed offers a versatile platform for the design of new materials, and has been applied to a number of areas[18]: for instance, the incorporation of antimicrobial, metal nanoparticles into bulk hydrogels has led to the development of skin grafts[19]; intrathecal delivery was demonstrated with nanoparticle-loaded hyaluronan NCs[20], and stimuli-responsive gels were achieved by the incorporation of nanoparticles that can respond to a magnetic field[21, 22] or irradiation[23, 24] and thus impact the characteristics of the whole matrix, such as the release of a payload[23] or the induction of mechanical forces able to actuate cells.[25] The myriad of properties achievable from the synergy of a hydrophilic matrix and nanoparticles have been discussed in a recent review[18].

In this work, we report the preparation and evaluation of a dual delivery matrix based on a nanocomposite gel of enzymatically cross-linked gelatin and embedded poly(3-hydoxybutyrate) nanoparticles. We have assessed the co-encapsulation of two hydrophilic and hydrophobic drugs: naproxen sodium, a non-steroidal anti-inflammatory drug (NSAID),[26] solubilised in the hydrogel matrix, and curcumin, a hydrophobic polyphenol derived from turmeric, which displays anti-oxidant, anti-inflammatory and anticancer properties,[27-29] and was loaded into the polymeric nanoparticles. Gelatin is a biopolymer, derived from the partial hydrolysis of collagen and widely used in biomedical applications due to its excellent biodegradability and biocompatibility.[30, 31] In this paper, it is cross-linked with the natural enzyme microbial transglutaminase (mTGase), which preserves the biocompatibility and immunogenicity of the original material [32]. For the nanoparticles, poly(3-R-hydroxybutyrate) (PHB) was used, the principal representative of bacterial polyhydroxyalkanoates (PHAs), which offer a competitive alternative to conventional synthetic polymers such as polypropylene, polyethylene and polyesters. These polymers are nontoxic and renewable. Their biotechnology output does not depend on hydrocarbon production and their biodegradation intermediates and resulting products (water and carbon dioxide) do not provoke adverse reactions in environmental media or living systems[33-35].

We evaluated the rheological properties of the nanocomposite gels and measured the combined release of the two drugs. Overall, the system we propose offers a simple, cheap

and sustainable approach to combination therapy with drugs displaying opposite solubility characteristics.

2. Experimental

2.1 Materials

Gelatin. Fish gelatin was donated by Rousselot, France. All experiments were carried out using a single batch of gelatin (from 2008). The gelatin was extracted from tilapia fish skin by an acidic process, and possesses an isoelectric point pI~ 8-9, bloom strength of 275, and a gelation temperature of ca. 23°C at the concentration used. The average molecular weight was determined as ~36 kDa ± 12% by GPC (Smithers Rapra). **Enzyme**. The cross-linker used in this study was microbial transglutaminase (mTGase), obtained from N-Zyme BioTec (Darmstadt, Germany) (specific activity: 1.6 U-mg solid; molecular weight: 38 kDa; purity >80%). The enzyme is stable between pH 5-9, and 0-40 °C. **Poly(3-hydroxybutyric acid)**. The polymer was donated by PHB Industrial S-A, Usina da Pedra s-n – Serrana, São Paulo, Brazil. The poly(-3-hydroxybutyric acid) is of natural origin, molecular weight around 600,000 (from GPC), melting point~ 170 °C, and negligible solubility in water. **Drugs**. Naproxen sodium, ≥ 98.0% purity (CAS number 26159-34-2) was obtained from Sigma-Aldrich and curcumin, 95% purity (CAS number 458-37-7, was obtained from Santa Cruz Biotechnology, USA. **Other chemicals.** Organic solvents: 2-propanol, acetone and dichloromethane were obtained from Sigma-Aldrich. Phosphate buffered saline and Tween 80 were obtained from Sigma-Aldrich. Pluronic F68 (F68) was obtained from MP Biomedicals.

Preparation of the Polymeric Nanoparticles (PNP)

2.2 Poly(3-hydroxybutyrate) (PHB) nanoparticles.

Nanoparticles were prepared by the solvent displacement method.[36, 37] PHB was solubilized in dichloromethane to a concentration of 5 mg mL^{-1}. This stock solution was added drop-wise under magnetic stirring at 800 rpm into an alcoholic solution (2-propanol containing 1% w/w of Tween 80). The system was left overnight in the fume hood under magnetic stirring to eliminate dichloromethane excess. The particles were centrifuged (8000 rfc) and the supernatant removed. The particles were dispersed in ultrapure water (18.2 MΩ

cm) and freeze-dried for at least 24 hours. The freeze-dried powder was kept in the freezer until use.

2.3 Curcumin-loaded Polymeric Nanoparticles

The physical encapsulation of curcumin was carried out using the solvent displacement method described above. Instead of the pure polymer, a PHB-curcumin solution was made in dichloromethane with a final PHB concentration of 5 mg mL^{-1} and 50 µg mL^{-1} of curcumin.

2.4 Entrapment efficiency (EE) and Drug loading (DL).

The amount of curcumin entrapped into the PNP was determined by measuring the amount of curcumin remaining in the supernatant after centrifugation. The difference between the initial amount and the free amount in the supernatant provides the entrapment efficiency of curcumin into the PNP. Drug concentration was measured in triplicates by a validated UV-Vis method. [38, 39]

Drug loading determination was performed through the dissolution of a known amount of freeze-dried powder in dichloromethane. The determination of the amount of curcumin in the PNP was performed as follows: 5 mg of the freeze-dried particles containing the drug was solubilized in 5 mL of dichloromethane and incubated at 37 °C for 2 hours in a sealed vial and covered with foil. The curcumin concentration was determined by UV–Vis using a standard calibration curve of curcumin prepared under identical conditions.

Preparation of the Gelatin-PNP Hydrogels

2.5 Gelatin-PNP dispersions.

Gelatin stock solutions were prepared as follow: 20% w/w gelatin in phosphate buffered saline (PBS) was left to swell overnight (pH 7.4) at 4 °C. Before use, the solutions were incubated in a water bath at 37 °C for 30 min.

Four samples with final composition of 10% w/w gelatin and 0, 1, 2 and 4% w/w PHB nanoparticles were prepared. Pluronic F68 was added into the sample containing the nanoparticles to improve the stability of the dispersion of the PNP in the gelatin and decrease aggregation. The final composition of F68 was 2% w/w. The samples were then placed in an ultrasonic bath for 10 minutes. The gelatin-PNP dispersions were prepared in triplicates.

2.6 Preparation of the Physical Nanocomposite Gels (PG)

Gelatin forms physical gels when lowering the temperature below the melting point (ca. 23°C for Tilapia gelatin) through the formation of triple-helices, which act as cross-links in the network[40]. To perform rheological measurements on the physical nanocomposite gels, the Gelatin-PNP dispersions were loaded onto the rheometer in the sol state at 25 °C (above melting temperature), and rheological measurements were started upon a step-decrease of the temperature to 21 °C (below melting temperature). The temperature was controlled by a Peltier unit (with a cooling rate of ca. 30 °C/min). The nomenclature used for the samples is: PG0, PG1, PG2 and PG4, to denote physical gels (PG) with 0, 1, 2 and 4% w/w of PHB particles.

2.7 Preparation of the Chemical Nanocomposite Gels (CG)

Chemical networks were formed in the presence of the enzyme mTGase, as described previously.[32, 40, 41] The required volume of mTGase stock solution was added to the previously gelatin-PNP dispersions to achieve a final enzyme concentration of 20 U mTGase/g of gelatin. Samples were rapidly vortex-mixed and then loaded onto the rheometer plate preheated at 37 °C. It is estimated that around one minute elapsed between the addition of mTGase to the sample and the start of the measurements. The nomenclature used for these samples is: CG0, CG1, CG2 and CG4, to denote chemical gels (CG) with 0, 1, 2 and 4% w/w of PHB particles.

Preparation of the dual release nanocomposite gels

2.8 Loading of naproxen sodium in the DDS

The nanocomposites all comprised 10% w/w gelatin and 4% w/w curcumin (CM)-loaded polymeric nanoparticles (prepared as described in section 2.3). Naproxen sodium (NS) was directly added into the gelatin-PNP dispersion before performing the enzymatic cross-linking. The sample was then placed in a mild ultrasonic bath for 10 minutes. Following this, the chemical networks were formed by adding the enzyme mTGase in a water bath at 37 °C. Figure 2 (c, d and e) schematically describes the preparation of the dual-drug-loaded nanocomposites gel, referred to as 'Gelatin(NS)-PNP(CM)'. These were prepared in triplicates for the *in vitro*-release study.

2.9 In-vitro co-sustained drug release

For *in-vitro* release studies, the Gelatin(NS)-PNP(CM) nanocomposite gels were transferred to a dialysis tube (1kDa cut-off) (Sigma Aldrich). The dialysis tube was sealed and immersed in

15 mL PBS:ethanol release medium (8:2 volume ratio), under continuous magnetic stirring at 37 ◦C. At specified time intervals, 0.2 mL of solution was drawn from the release medium and replaced by 0.2 mL fresh PBS:ethanol solution. The amount of drugs in the release medium was quantified by UV-Vis spectroscopy using calibration curves established with the same solvent composition.

Characterization

Rheology. Rheological measurements were carried out on a strain-controlled ARES rheometer (TA Instruments) using parallel plate geometry (25 mm diameter) and the temperature was controlled by a Peltier unit (± 0.1 °C). To prevent loss of solvent due to evaporation, we applied a thin layer of paraffin oil around the samples, and geometry-covers were placed around the plates. To study the build-up of the storage (G') and loss (G'') moduli upon gelation, time sweep-cure experiments were performed at a fixed frequency of 6.28 rad/s and strain of 1%. The gel point was established as the time when G' reaches the arbitrarily defined value of 1 Pa [42].

Dynamic light scattering (DLS). DLS measurements to determine the average size and size distribution of the nanoparticles were performed using a Nanosizer 90ZS (Malvern Instruments, Southborough, MA). All data analysis was performed in automatic mode and samples measured in triplicates.

Scanning electron microscopy (SEM). The size and morphology of the PHB nanoparticles were evaluated by scanning electron microscopy (Zeiss LEO 440). Freeze-dried nanocomposite chemical gels with 10% w/w gelatin and 0, 1, 2, 4% w/w PHB nanoparticles were prepared for scanning electron microscopy measurements. Discs of freeze-dried gels were cut off and mounted onto stubs using double-sided carbon tape. Gold was coated in a sputter coater and the cross-section morphologies of the nanaocomposite gels were examined using a Fei Quanta 200 Field Emission Gun.

UV–Vis spectroscopy. The release of the drugs from the nanocomposite gels was performed with UV-Vis spectroscopy (Perkin Elmer UV-Vis Spectrophotometer Lambda 2) at the absorbance maximum: 432 nm (curcumin) and 231 nm (naproxen sodium). Calibration curves were constructed in the relevant medium to measure entrapment efficiency, drug loading and drug release.

Release mechanism studies. To describe the release behaviour, an empirical power law equation proposed by Peppas et al.[43, 44] was used:

$$\frac{M_t}{M_\infty} = kt^n \quad (1)$$

$$log\left(\frac{M_t}{M_\infty}\right) = logk + n\log\ t \quad (2)$$

where M_t and M_∞ are the absolute cumulative amount of drug released at time *t* and infinite time, respectively, *k* is a constant incorporating structural and geometric characteristics of the device, and *n* is the release exponent, indicative of the mechanism of drug release.

3. Results and discussion

3.1 Preparation of Gelatin-PNP nanocomposite gels

The PHB nanoparticles were prepared by the solvent displacement method.[36, 45] This method is based on the precipitation of a preformed polymer from an organic solution and the diffusion of the organic solvent in the medium. The medium can be an aqueous solution (with or without surfactants) or an organic solution (provided it is not a good solvent for the preformed polymer).[37] Figure 1 shows SEM images of the PHB nanoparticles prepared by this method. The inset presents the distribution of sizes obtained from DLS measurements, showing an average size of 216 nm with a size distribution from 164 to 295 nm, corresponding to a polydispersity of 11%.

The nanocomposites were prepared from gelatin, freeze-dried nanoparticles and F68 (1%) in PBS solution, as described in the experimental section. Figure 2 shows the appearance of the gelatin-PNP hydrogels with increasing amounts of particles prepared from the chemical protocol. Figure 2(a) shows the appearance of the gelatin-PNP nanocomposite gels in the hydrated state, straight after preparation and Figure 2(b) shows the same samples after the freeze-drying process. Sample 5 is the drug loaded nanocomposite gel, Ge nanocomposites of gelatin and poly(3-hydroxybutyrate) nanoparticles for dual drug release latin(NS)-PNP(CM), which was further tested for in vitro drug release.

Figure 3 reports the evolution of the storage (G') and loss moduli (G'') as a function of PHB nanoparticle-loading of the nanocomposite gels prepared from the enzymatic cross-linking process (chemical protocol). The time sweeps show a relatively sharp increase of both moduli

over the first few minutes of the measurements, reflecting the cross-linking process, followed by a near-plateau region, where only a very slight increase in mechanical properties is observed. Both time and frequency-sweeps measurements reveal some differences with particle loading. Gelatin also displays a natural, temperature-induced gelation process, which occurs through the formation of triple-helices (reminiscent of the native collagen), which act as junction points for the network. For comparison purposes, time and frequency sweep measurements of the gelatin-PNP nanocomposites obtained from the physical gelation process are shown in supporting information (Figure S1).The storage modulus (G') and gelation times after 180 min for samples prepared by the physical and chemical protocols are shown in Table I. The storage modulus shows a dependence on the amount of PNP embedded in the matrix: the higher the amount of PNP, the higher the value of G', reflecting increasing elasticity of the gels with particle loading. The introduction of PHB nanoparticles at 1% w/w induces a slight increase in the storage modulus, when compared with the pristine gelatin. A further increase in the concentration of nanoparticles (up to 4% w/w) increases the storage modulus to a larger extent, both for the physical and the chemical protocols, while not affecting the solid-like *vs* liquid-like properties (tan (δ)). This increase in mechanical properties by the introduction of nanoparticles is not surprising [46] and may result from an increase in connectivity arising from gelatin-particles interactions or an increased cross-linking between gelatin chains, caused by a higher effective concentration due to the presence of particles (either enhancing triple-helix formation for the physical gels, or covalent bonding from the chemical process). The presence of the nanoparticles also reduces the gelation time, down to <30% of the original time at the highest loading for the physical gels and ca. 44% for the chemical gels (Table I). Overall, the nanoparticles have a similar effect on the gel properties, using either type of gelation protocol (chemical or physical). For practical applications however, the enzymatic cross-linking process is the only viable process and leads to gelation times (below 10 min), which are suitable for injectable gels. Therefore, in the following, we focus on the evaluation of the enzymatic cross-linking process.

3.2 Microstructure of the gelatin-nanoparticles hybrid gels -Chemical protocol

Information on the structure was obtained from electron microscopy on the freeze-dried gels, with and without nanoparticles. Figure 4(a) shows the micrographs of the gelatin sample (CG0), which consists in a lightly etched surface with hollow arrays interconnected by thin walls. This structure is characteristic of the morphology of pristine gelatin.[47]

The presence of PNP disrupts the native structure of the gelatin microstructure. Each concentration of the PNP provides different structures. Figures 4(b), 4(c) and 4(d) show the morphology from samples CG1, CG2 and CG4, respectively. With 1 % w/w PNP (CG1), it is still possible to see the lightly etched gelatin microstructure, as shown in Figure 4(b). At the highest loading of 4 % w/w PNP, CG4 appears quite different from the other samples. Figure 4(d) shows a highly porous gelatin–PNP hybrid, which presents a heterogeneous surface texture with large pores and interconnections. Porous structures allow the water to be expelled or absorbed by convection, a much faster process than by diffusion.[48] This structure formed by the combination of a gel matrix with nanoparticles may explain the increase in storage modulus when compared to pristine gelatin. Agglomerates of PNP were observed in the cross-sectional areas in all samples. The presence of segregated PNP is mainly observed in CG1 and CG2, which are shown in Supplementary Figure S3, S4 and S5.

In vitro Drug Release Studies

Following the encapsulation of both drugs, *in vitro* co-sustained release from the nanocomposite was measured. Curcumin was encapsulated in the PNP with 52% entrapment efficiency, whereas naproxen sodium was directly incorporated in the gelatin matrix. Simultaneous detection of naproxen and curcumin released in the medium from the nanocomposite gels was performed by UV-Vis absorbance, since their absorbance maxima are well separated.

Figure 5(a) shows the sustained release of naproxen from the gelatin-PNP hydrogel. An initial burst release was observed in the first hours of the measurement, and around 90% was released on the third day. The released from the gel was much slower than the free drug diffusing through the membrane, which was complete in less than 24 hours.

Figure 5(b) compares the sustained release of curcumin from the PNP, either in the gel matrix or in solution. An initial burst release of curcumin was observed in both samples. In the free PNP, over 82 % of curcumin was released after 48 hours (red curve). The hybrid system (gelatin-PNP, black curve) provides a delay in drug release compared to the free PNP. After four days of measurement, over 90% of CM was released from the system containing PNP only, whereas only ca. 40% was released when the PNP are embedded in the gel matrix. After seven days, still only 52% of curcumin was released, while naproxen release was complete

between the third and fourth day. Thereby, the hydrogel further delays the release from the nanoparticles that are embedded in the matrix.

The release profile was analysed by the model described by Peppas[43, 44, 49]. The kinetic constant and release exponent obtained from fitting the curves to this model are shown in Supplementary Table SI. The release of curcumin from the free PNP proceeds via an anomalous transport process, whereby the drug is released by a complex mechanism, namely, a mixture of Fickian diffusion and surface degradation of the matrix. However, in the presence of gelatin, the release of curcumin follows a Fickian diffusion profile. For naproxen, the release exponent indicates an anomalous transport model. In the gelatin sample, besides drug diffusion, surface degradation is a heterogeneous process in which degradation (and subsequent erosion) is confined to a thin surface layer of the biopolymer[49].

Applicability of the nanocomposites as an injectable, dual-delivery matrix

In order to assess the potential of the nanocomposites as injectable gel formulations, a preliminary evaluation of their storage as a frozen formulation was performed. The gelatin-PNP dispersion (1% PNP) was kept in the freezer at -20 °C over a period of several days. The sample was then warmed up to 37◦C in a water bath, and mTgase (20 U/g) quickly added and mixed into the gelatin-PNP dispersion (Supporting Information, S2). The chemical nanocomposite gel presented the same rheology profile than a freshly prepared nanocomposite gel (SI), with only a small decrease in the storage modulus, which remained of the same order of magnitude (ca. 3,5 kPa). Injection with a hypodermic syringe was also performed as proof-of-concept, following rapid mixing of mTGase and gelatin solutions, and showed that the viscosity of the pre-gelation mixture was suitable for injection, *i.e.* did not required excessive pressure.

Our previous results on the gel matrices using F-actin staining (cell spreading and morphology), cell compatibility (LIVE/DEAD assay) and proliferation (MTS) assays on MC3T3 osteoblast cell lines[41], as well as cell compatibility (AlamarBlue) and proliferation assays (MTS/MTT assays) on rat bone-marrow mesenchymal stem cells (rBMSCs)[32] showed that the crosslinked gelatin matrix was not cytotoxic and supported cell proliferation. PHB based micro- and nano- carriers have been reported for the delivery of various drugs; PHB is degraded *in vivo* through both enzymatic and non-enzymatic processes under normal conditions [50, 51, 52]. *In vivo* testing of the nanoparticles will be the subject of future work.

Conclusions

A dual drug delivery system that can load and release different drugs in a controlled manner is a strategy that has been attracting increased interest over the past few years. The nanocomposite reported in this study, comprising curcumin-loaded PHB nanoparticles embedded in a gelatin matrix with solubilised naproxen sodium, provides a sustainable, cheap and simple approach. Two gelation processes were evaluated: physical gelation (due to the formation of triple helices at low temperatures) and chemical cross-linking (obtained with the enzyme transglutaminase, which thus does not compromise the biocompatibility of the original material). The incorporation of PHB nanoparticles in the gelatin matrix did not interfere with the formation of the gel. Increasing the concentration of nanoparticles (from 1 to 4 % w/w) resulted in an increase in storage modulus and a decrease in gelation time, with both the physical and chemical protocols. Scanning-electron microscopy images showed that the presence of PNP disrupted the native structure of the gelatin chains, showing a heterogeneous surface texture with large pores and interconnections.

Curcumin was successfully loaded into the PHB nanoparticles, with an entrapment efficiency around 52%, while naproxen sodium was concomitantly solubilised in the gelatin matrix. *In vitro* studies of the co-sustained release of naproxen and curcumin from the nanocomposite gels obtained from the chemical cross-linking process showed that the hydrogel matrix acts as a physical and chemical barrier delaying the diffusion of both drugs. After four days, only 40% of curcumin was released from the hybrid gels, compared to 82% from the free PNP.

The gelatin-PNP dispersion can be frozen, warmed-up and mixed with transglutaminase, displaying the same rheological properties as a freshly prepared sample, and is easily injectable through a hypodermic syringe. Based on the demonstrated biocompatibility of the starting materials, this work opens up the possibility of using the nanocomposites as in-situ gelling, injectable formulations, for instance using a double-barrel syringe. These soft nanocomposites offer simultaneous, sustained release for both a hydrophobic and a hydrophilic drug over a long period of time, and thus present potential as a sustainable, dual drug-loading matrix.

Acknowledgements

This manuscript was supported by FAPESP (grants 07/07919-9 and 13/01284-2) and CNPq Brazilian Agencies.

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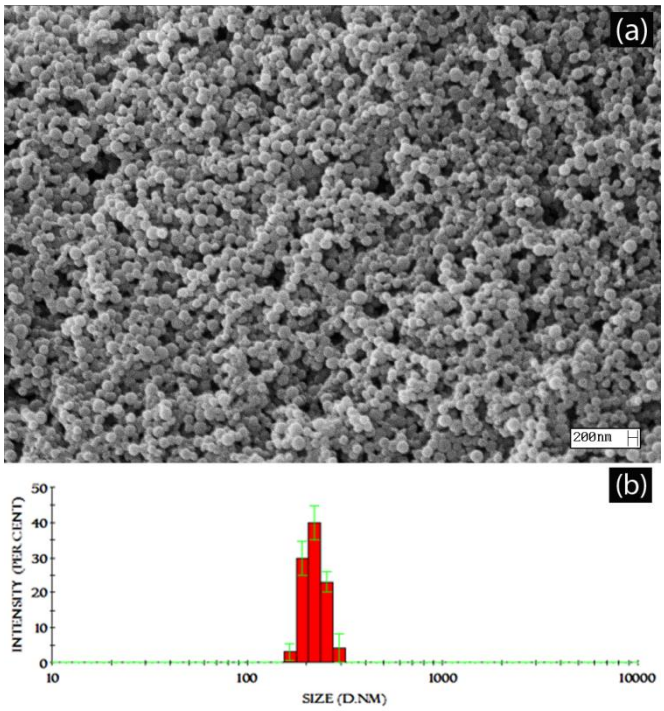

Figure 1. (a) SEM microphotographs of the PHB nanoparticles prepared by the solvent displacement method. Scale bar: 200 nm. (b) Size distribution by intensity obtained from DLS.

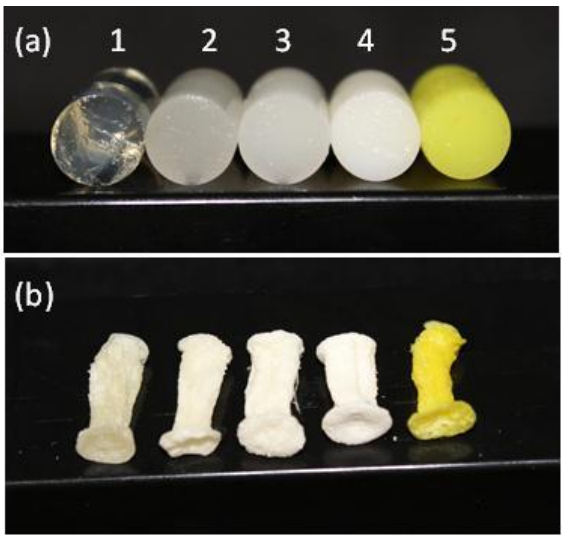

Figure 2. Pictures showing the appearance of the gelatin-PNP chemical gels containing 10% w/w gelatin and 4% w/w polymeric nanoparticles loaded with curcumin: (a) at room temperature and (b) after freeze-drying. Samples code: 1: CG0, 2: CG1, 3: CG2, 4: CG4 and 5: nanocomposite CG4 loaded with naproxen sodium and curcumin.

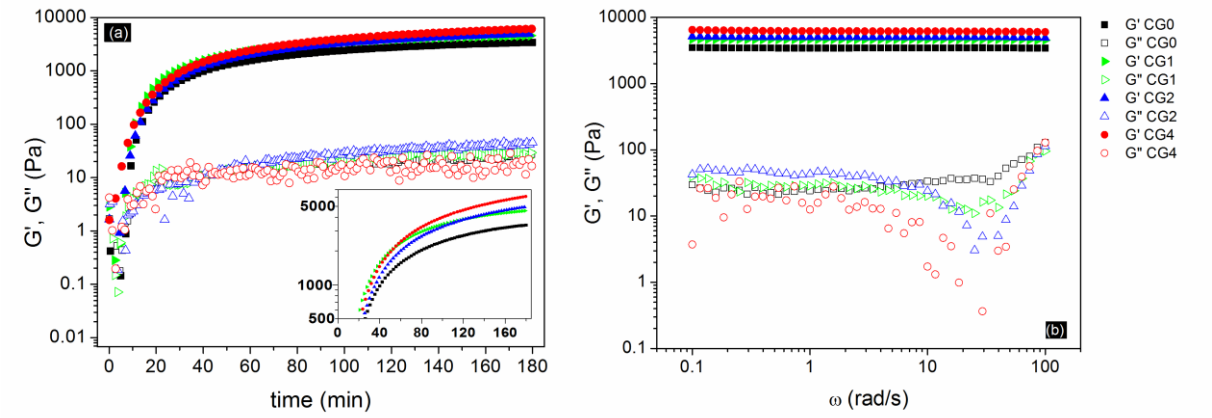

Figure 3. (a) Time and (b) frequency sweep measurements of the nanocomposite gels prepared from the chemical protocol (CG) with 10 (CG0), 20 (CG1), 30 (CG3) and 40 (CG4) mg of nanoparticles /g of gelatin. The inset in (a) shows an expanded part of the time sweep measurements to highlight differences at varying PNP loadings. The storage modulus (G') is represented by filled symbols and loss modulus (G'') with open symbols.

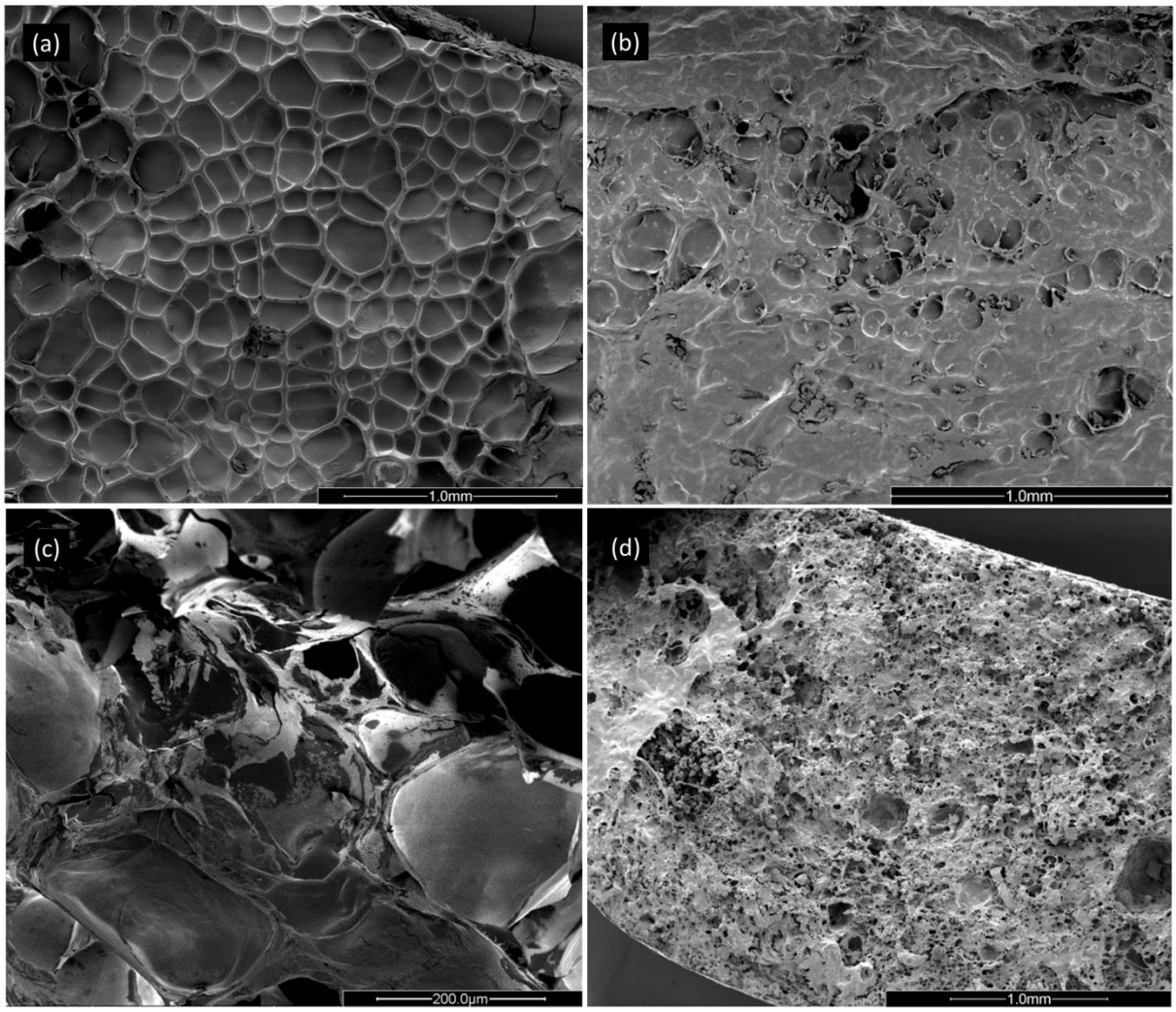

Figure 4. Microstructure of the gels obtained from electron microscopy on the freeze-dried samples. (a) CG0 (pure gelatin); and gelatin-nanoparticles hybrid gels: (b) CG1; (c) CG2; (d) CG4.

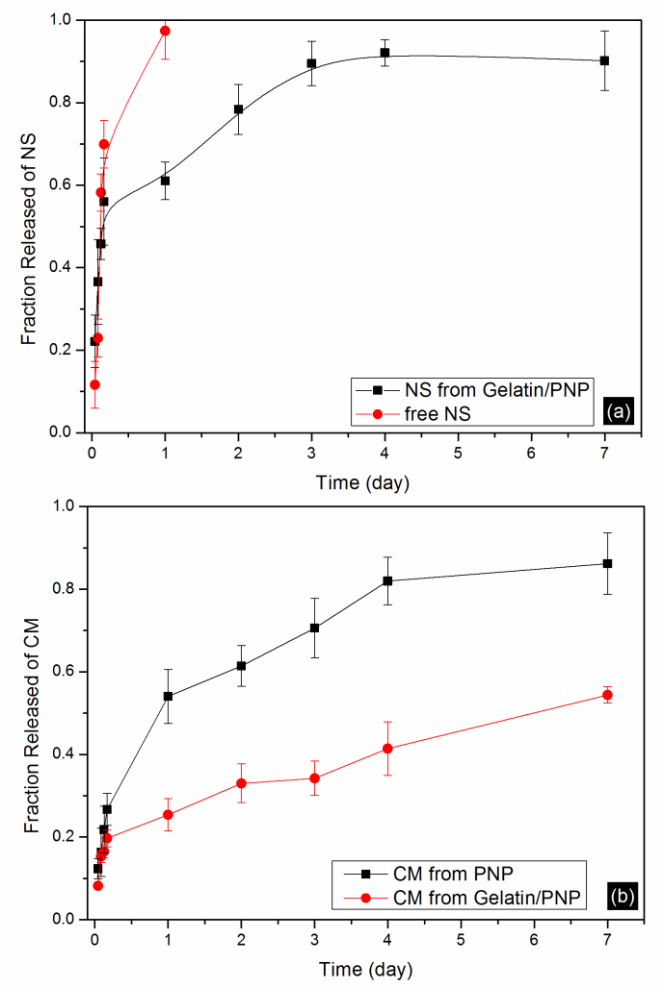

Figure 5. Sustained release of (a) naproxen sodium and (b) curcumin from the Gelatin-PNP hydrogel obtained by enzymatic cross-linking (black dots) and from the PNP (red dots). Lines are guides to the eye.

Table I. Values of the final storage modulus and gelation times of the gelatin-PNP hybrid gels submitted either to a physical (PG) or a chemical cross-linking process (CG).

network type	samples	G' a(Pa)/ SDb(%)	tan (δ)	t gelation (min)
Physical Protocol	PG0	1935/5.4	0.03596	1.4
	PG1	2285/3.5	0.03937	0.8
	PG2	2631/8.5	0.03357	0.5
	PG4	3189/4.8	0.04366	0.4
Chemical Protocol	CG0	3434/1.0	0.00725	8.3
	CG1	4607/1.0	0.00653	5.2
	CG2	5357/7.3	0.00922	4.8

	CG4	6220/9.1	0.00360	3.7

a. Mean G’ value of three measurements; b. SD = standard deviation relative to the mean; c. mean of three measurements.